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Hyperhomocysteinaemia is associated with coronary events in type 2 diabetes

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Abstract. Becker A, Kostense PJ, Bos G, Heine RJ, Dekker JM, Nijpels G, Bouter LM, Stehouwer CDA (VU University Medical Center, Amsterdam, the Netherlands). Hyperhomocysteinaemia is associated with coronary events in type 2 diabetes. *J Intern Med* 2003; **253:** 293–300.

Objectives. Amongst nondiabetic individuals, a high serum homocysteine concentration is an independent but relatively weak risk factor for coronary events. However, it is not known whether homocysteine increases risk of coronary events in type 2 diabetes. Therefore, we examined the combined effect of homocysteine and type 2 diabetes on risk of fatal and nonfatal coronary events.

Subjects. We assessed the 10-year risk of coronary events associated with homocysteine amongst diabetic ($n = 140$) and nondiabetic ($n = 361$) individuals.

Design. We did this in the Hoorn Study, a population-based study of glucose tolerance and related complications in Caucasian men and women aged 50–75 years.

Results. The incidence rate for coronary events was 2.63 (29 of 140) per 100 person-years amongst diabetic and 1.29 (42 of 361) amongst nondiabetic individuals. Amongst diabetic individuals, risk of coronary events increased 28% for each 5-μmol L^{-1} increment of homocysteine (hazard ratio, 1.28; 95% CI, 1.02–1.58). This risk was independent of age, sex, hypertension, total cholesterol, HDL-cholesterol, cigarette smoking, body mass index and glomerular filtration rate. In nondiabetic participants, homocysteine was not associated with an increased risk of coronary events (hazard ratio for each 5-μmol L^{-1} increment of homocysteine, 0.86; 0.52–1.41).

Conclusions. These data suggest that homocysteine is significantly associated with coronary events in individuals with type 2 diabetes, independent of traditional cardiovascular risk factors. Investigation of the effect of treatment with vitamin B on prognosis of individuals with type 2 diabetes is warranted.

Keywords: cardiovascular disease, coronary artery disease, diabetes, folic acid, homocysteine, prevention.

Introduction

Amongst nondiabetic individuals, hyperhomocysteinaemia is an independent risk factor for cardiovascular and all-cause mortality. In addition, it increases risk of fatal and nonfatal coronary events [1, 2], although the association with coronary events is relatively weak [3].

Type 2 diabetes causes excess cardiovascular morbidity and mortality, predominantly as a result of coronary events [4]. The increased cardiovascular risk in type 2 diabetes is not fully explained by conventional cardiovascular risk factors. Alternative risk factors, such as homocysteine, may partly explain this increased risk. Prospective data concerning this issue are scarce. In previous studies, homocysteine was found to be a strong risk factor for all-cause and cardiovascular mortality in type 2 diabetes [5–7]. However, there are no data on the (disease-specific) association of homocysteine with coronary events amongst individuals with type 2 diabetes. If homocysteine concentration is associated

with coronary events in type 2 diabetes, lowering homocysteine concentration may be an additional target for the prevention of fatal and nonfatal coronary events amongst individuals with type 2 diabetes.

In view of these considerations, the present study assessed the combined effect of hyperhomocysteinaemia and type 2 diabetes on the risk of coronary events during 10 years of follow-up. We addressed these issues in the Hoorn Study, which was specifically designed to examine type 2 diabetes as a cardiovascular risk factor [8].

Materials and methods

Study population

The Hoorn Study is a population-based cohort study on glucose intolerance and other cardiovascular risk factors in a 50- to 75-year-old-general Caucasian population, which started in 1989. The study population has been described in detail before [8].

Briefly, 2484 individuals (71% of those invited) participated. All participants, except previously diagnosed as diabetic individuals treated with oral glucose-lowering agents or insulin, underwent an oral glucose tolerance test (OGTT) according to the WHO guidelines [9]. For reasons of efficiency, participants with a 2-h postload glucose ≥ 7.5 mmol L^{-1}, all participants with type 2 diabetes and a random sample of participants with a 2-h postload glucose <7.5 mmol L^{-1}, stratified by age and sex, were invited within 4 weeks for a second visit to investigate glucose-intolerance-related complications [709 invited, of whom 631 (89%) participated]. These participants underwent a second OGTT (except those who already used blood-glucose-lowering agents; $n = 67$). Participants in the present study population thus represented a stratified random sample of all individuals with normal or impaired glucose tolerance or type 2 diabetes in the initial cohort [8].

The Ethical Review Committee of the VU University Medical Center approved the Hoorn Study. Informed consent was obtained from all participants.

Laboratory and clinical assessments

After an overnight fast, blood was drawn from an antecubital vein. We measured serum total homocysteine, glucose, HbA_{1c}, creatinine, total cholesterol, HDL-cholesterol and triglycerides. Concentrations of serum total (free plus protein-bound) homocysteine were assessed in frozen (−20 °C) Ethylene diaminetetraacetic acid (EDTA) serum by using high-performance liquid chromatography with fluorescence detection [10]. The intra- and inter-assay coefficients were 2.1 and 5.1%, respectively. Serum total cholesterol, HDL-cholesterol and triglyceride levels were measured by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany) and serum creatinine by means of modified Jaffé method [5]. We estimated the glomerular filtration rate by the method of Levey *et al.* which is thought to be more accurate than the Cockcroft–Gault formula [11–13]. Blood pressure was measured as the mean of four measurements performed on two different occasions, by means of a random-zero sphygmomanometer under standardized conditions. Hypertension was defined according to the criteria of the World Health Organization (at time of data collection) as follows: a systolic blood pressure of ≥160 mmHg and/or a diastolic blood pressure of ≥95 mmHg, and/or the use of antihypertensive medication [14]. Use of more recent criteria, i.e. systolic blood pressure ≥140 mmHg and/or diastolic blood pressure of ≥90 mmHg [15], for hypertension, did not influence the results (data not shown). Participants were classified as current cigarette smokers, nonsmokers or former smokers. We calculated body mass index (BMI) and waist-to-hip ratio as previously described [8]. Finally, we obtained an ankle–brachial blood pressure index and a resting electrocardiogram. Participants were classified as having cardiovascular disease when they had a positive history of myocardial infarction and/or had an electrocardiogram with a Minnesota code 1.1–1.3, 4.1–4.3, 5.1–5.3 or 7.1 and/or had undergone coronary bypass surgery or angioplasty, and/or had an ankle–brachial blood pressure index of less than 0.9 in either leg and/or had undergone a peripheral arterial bypass or amputation.

Follow-up

The population register of the city of Hoorn provided the vital status of participants until 1 January, 2000. Causes of death were extracted from the medical records of the general practitioner and the local hospital. Information about nonfatal coronary events was obtained by reviewing medical records of

the hospital of Hoorn. Of 24 individuals who moved out of town, we obtained information on (non) fatal events from the new local municipalities, general practitioner and hospital. A total of 130 individuals of 631 candidate participants could not be included because they refused permission for their records to be reviewed (amongst these 25 who moved). We coded causes of death and nonfatal events, as diagnosed by the doctor and as written in the medical records, according to the International Classification of Diseases, Injuries, and causes of Death, ninth revision (ICD-9) [16]. If a participant died within 28 days after a nonfatal coronary event, the event was classified as fatal. Fatal and nonfatal coronary events were defined as ICD-9 codes 410–414 ('ischaemic heart diseases: myocardial infarction, angina pectoris and other manifestations of ischaemic heart disease').

Statistical analyses

For all analyses we used SPSS 10.1 for Windows. Data are presented as mean (±standard deviation) or median (interquartile range). Differences between groups in continuous variables were tested with Student's *t*-test; in case of a skewed distribution with the Mann–Whitney test; and in case of percentages with the chi-square test. *P*-values were based on two-sided tests and were considered statistically significant if <0.05. Linear regression analysis was used to assess baseline associations between homocysteine concentration and cardiovascular risk factors.

We used Cox regression to estimate hazard ratios of coronary events as a function of serum homocysteine concentration. Analyses were stratified for the presence of diabetes [5, 17]. In addition, we used Cox regression to estimate the effects of cardiovascular risk factors on coronary events. Coronary events were defined as the first fatal or nonfatal coronary event during follow-up. Individuals with missing values for fatal or nonfatal events were omitted from the analyses. We calculated hazard ratios for homocysteine as a continuous variable, expressed per 5 μmol L^{-1} increment of homocysteine, and as a categorical variable (to allow for a nonlinear dose–response relation) [5, 18]. In the analyses with homocysteine as a categorical variable, we computed hazard ratios for homocysteine divided in four categories (≤9, 9.1–14.0, 14.1–19.0 and >19.0 μmol L^{-1}, respectively) [5, 18]. To test for trend, the four categories were added to the analyses as an ordinal variable.

Serum homocysteine concentrations were measured only at baseline. As a result of long-term within-person variability, a single measurement will lead to underestimation of the true association between the long-term average homocysteine concentration and disease rates [19]. Therefore, we adjusted hazard ratios for this so-called 'regression dilution bias' using a regression equation calculated by Clarke *et al.* on the basis of regression dilution ratios (RDR) of four prospective studies with repeated homocysteine measurements. The RDR was calculated as 0.89–0.03T. T stands for years between measurements; thus after 9.8 years of follow-up in the present study, the RDR would be 0.60 [20]. We also calculated the RDR in a sample ($n = 72$) of nondiabetic individuals of the Hoorn Study in whom homocysteine was remeasured 9.5 years after the initial examination. We found an RDR of 0.70, which is of a similar magnitude as the value calculated above. In the present analyses, we chose to use the RDR of Clarke *et al.* because this was based on a much greater number of individuals [20], and to set T at 4.9 (i.e. halfway through the follow-up period [19]; thus RDR = $0.89 - 0.03 \times 4.9 = 0.74$). Hazard ratios therefore represent risks associated with 4.9-year average homocysteine concentration, which is probably more useful than risks associated with homocysteine concentration at baseline. Hazard ratios displayed in Table 2 are thus corrected for regression dilution bias. (Uncorrected hazard ratios can be easily back-calculated by taking the corrected hazard ratio to the power of 0.74; for example, when the corrected hazard ratio is 1.25, the uncorrected hazard ratio is $1.25^{0.74} = 1.18$) *P*-values are not influenced by this correction.

In the first regression model, we calculated hazard ratios adjusted for the stratification variables age and sex. In the second model, we additionally adjusted for other major classical risk factors for cardiovascular disease, namely, total cholesterol, HDL-cholesterol, hypertension, cigarette smoking and BMI [21]. Potential confounders measured on a continuous scale were used as such in the regression analyses, except for HDL-cholesterol and BMI, because their association with coronary events was nonlinear. Therefore, HDL-cholesterol

concentration was defined as low if <0.9 mmol L^{-1} [22], and BMI was defined as high if >27 kg m^{-2} in men and >26 kg m^{-2} in women [23]. We computed product terms (such as diabetes × homocysteine) to assess whether homocysteine interacted with any cardiovascular risk factor or with time.

Results

Characteristics of the participants

At baseline, the cohort consisted of 174 diabetic and 457 nondiabetic individuals. Thirty-four diabetic and 96 nondiabetic individuals had missing values for nonfatal events and were excluded. Persons who were excluded did not differ from persons who were not excluded with regard to baseline characteristics (data not shown). Table 1 shows baseline characteristics and hazard ratios of coronary events associated with cardiovascular risk factors. Of all type 2 diabetic individuals, 73 (52.1%) were newly detected and 67 (47.9%) were known to have diabetes. The median duration of diabetes amongst individuals with previously diagnosed diabetes was 6.2 (interquartile range: 2.6–11.5) years. After up to 10 years of follow-up (median, 9.8; range, 0.5–11.2 years), 50 amongst 140 diabetic individuals had died (three died as a result of coronary events) versus 57 amongst 361 nondiabetic individuals (six died as result of coronary events). During follow-up 29 amongst 140 diabetic individuals had one or more nonfatal coronary events as opposed to 42 amongst 361 nondiabetic individuals. Of the excluded 130 individuals, 12 persons died due to an unknown cause. Table 2 displays baseline associations between homocysteine and cardiovascular risk factors.

Coronary events

The incidence rate of coronary events per 100 person-years was 2.63 (31 cases amongst 140 individuals during 1177 person-years) amongst diabetic and 1.29 (47 cases amongst 361 individuals during 3631 person-years) amongst nondiabetic individuals (Table 3).

Table 3 shows that, amongst diabetic individuals, homocysteine was significantly associated with 10-year risk of coronary events. After adjustment for other cardiovascular risk factors, the risk of coronary events rose with 1.28 (95% CI, 1.02–1.58) for each 5 μmol L^{-1} increment of serum homocysteine. Per category increment of homocysteine, the hazard ratio was 1.55 (CI, 0.84–2.86); after adjustment for confounders, it was 1.75 (CI, 0.92–3.36) (Fig. 1).

Table 1 Baseline characteristics and hazard ratios of coronary events associated with cardiovascular risk factors

	Nondiabetic individuals (n = 361)	Diabetic individuals (n = 140)	P-value[a]	Difference in risk factor	Coronary events RR (95% CI)[b]
Male gender (%)	51.0	47.1	0.59	Yes versus no	1.25 (0.79–1.95)
Age (years)	63.4 (7.1)	65.7 (6.8)	0.001	Per year increase	1.04 (1.01–1.08)
Total homocysteine (μmol L^{-1})[c]	11.3 (9.4–14.1)	11.2 (9.1–13.6)	0.41	Per μmol L^{-1} increase	1.02 (0.99–1.05)
HbA_{1c} (%)	5.4 (0.5)	7.2 (1.9)	<0.001	Per 1% of haemoglobin	1.04 (0.87–1.24)
Body mass index (kg m^{-2})	26.6 (3.5)	28.6 (4.5)	<0.001	High versus low	0.67 (0.42–1.06)
Smoker (no/yes/former) (%)	36.3/26.0/37.7	42.0/22.5/35.5	0.48	Yes versus no	2.15 (1.16–4.01)
				Former versus no	1.72 (0.94–3.14)
Systolic blood pressure (mmHg)	137.0 (19.3)	144.3 (19.0)	<0.001	Per mmHg increase	1.01 (1.0–1.02)
Diastolic blood pressure (mmHg)	82.3 (10.0)	83.0 (10.4)	0.49	Per mmHg increase	1.00 (0.98–1.02)
Hypertension (%)	32.4	54.3	<0.001	Yes versus no	2.09 (1.32–3.32)
Total cholesterol (mmol L^{-1})	6.7 (1.1)	6.4 (1.3)	0.013	Per mmol L^{-1} increase	1.22 (1.02–4.47)
HDL-cholesterol (mmol L^{-1})	1.3 (0.4)	1.1 (0.3)	<0.001	High versus low	0.59 (0.27–1.27)
Triglycerides (mmol L^{-1})[c]	1.5 (1.1–2.1)	1.9 (1.3–2.9)	<0.001	Per 10% increase	1.88 (1.22–2.88)
Glomerular filtration rate (mL min^{-1})[d]	67.8 (11.3)	68.2 (14.3)	0.80	Per mL min^{-1} increase	0.99 (0.97–1.01)
Prior cardiovascular disease (%)	18.8	33.6	<0.001	Yes versus no	3.17 (1.98–5.07)

Data are presented as mean (SD), percentage of the total or median (interquartile range); [a]diabetic versus nondiabetic individuals; [b]adjusted for age, sex and diabetes, except when this was the variable under consideration; [c]median (interquartile range); [d]estimated by the method of Levey *et al.* [11]; n = number; HDL cholesterol, high-density-lipoprotein cholesterol; HR, hazard ratio; CI, confidence interval; because of a non-linear association with coronary events HDL-cholesterol was entered above versus below 0.9 mmol L^{-1}, body-mass index above versus below 27 kg m^{-2} for men and above versus below 26 kg m^{-2} for women and triglycerides per 10% increase.

Table 2 Homocysteine concentrations: baseline associations with cardiovascular risk factors

	β[a]	SE(β)[a]	*P*-value
Gender (Male versus female)	0.12	0.029	<0.001
Age (per 1 year increase)	0.011	0.002	<0.001
Diabetes (Yes versus no)	−0.049	0.032	0.13
HbA_{1c} (Per 1% of haemoglobin)	0.0038	0.013	0.77
Body mass index (per 1 kg m^{-2} increase)	0.0019	0.004	0.61
Smoker			
Yes versus no	0.083	0.037	0.026
Former versus no	0.0089	0.017	0.60
Systolic blood pressure (Per 1 mmHg increase)	0.0012	0.001	0.092
Diastolic blood pressure (Per 1 mmHg increase)	0.0011	0.001	0.39
Hypertension (Yes versus no)	0.041	0.028	0.14
Total cholesterol (Per 1 mmol L^{-1} increase)	0.011	0.011	0.13
HDL cholesterol (Per 1 mmol L^{-1} increase)	−0.054	0.039	0.17
Triglycerides (Per 1 mmol L^{-1} increase)	0.016	0.011	0.13
Glomerular filtration rate (Per 1 mL min^{-1} increase)[†]	−0.01	0.001	<0.001
Prior cardiovascular disease (Yes versus no)	0.065	0.031	0.039

[a]Regression coefficient (β), standard error [SE(β)] and *P*-value obtained by linear regression analyses with homocysteine concentration (log-transformed because of a better fit of the regression model) as dependent and risk factors as independent variable, all adjusted for age, sex and diabetes (unless this was the variable under consideration); [†]estimated by the method of Levey *et al.* [11].

Table 3 Hazard ratios (95% confidence intervals) for coronary events associated with homocysteine during 10 years of follow-up

	Coronary events	
Individuals	HR[a]	HR[b]
Nondiabetic individuals		
Total homocysteine (Per category increment)	0.84 (0.49–1.44)	0.76 (0.44–1.31)
Total homocysteine (Per 5 μmol L^{-1} increment)	0.91 (0.57–1.44)	0.86 (0.52–1.41)
Diabetic individuals		
Total homocysteine (Per category increment)	1.55 (0.84–2.86)	1.75 (0.92–3.36)
Total homocysteine (Per 5 μmol L^{-1} increment)	1.25 (1.02–1.53)	1.28 (1.02–1.58)

HR, hazard ratio; [a]adjusted for age and sex; [b]adjusted for age, sex, hypertension, total cholesterol, HDL-cholesterol, body mass index and smoking.

Amongst nondiabetic individuals, homocysteine was not significantly related to risk of coronary events (Table 3). Hazard ratios per 5 μmol L^{-1} increment of serum homocysteine thus differed between individuals with and without diabetes (hazard ratios, 1.28; CI, 1.02–1.58 vs. 0.86; CI, 0.52–1.41, respectively). However, the test for interaction was nonsignificant (*P*-value of the product term diabetes × homocysteine was 0.29 and for diabetes × homocysteine in four categories, 0.22). In addition, we did not observe significant interactions between serum homocysteine and cardiovascular risk factors other than diabetes. We also did not find a significant time-to-event dependency for the association between homocysteine and coronary events in diabetic or in nondiabetic individuals. Therefore, the proportional hazards assumption is satisfied.

Additional analyses

After additional adjustment for prior cardiovascular disease at baseline [13], the association between homocysteine and coronary events amongst diabetic individuals became borderline significant (HR, 1.22; 95% CI, 0.97–1.54). However, adjustment for this variable may amount to overadjustment, as cardiovascular disease most likely is an intermediate factor in the causal pathway linking homocysteine to coronary events [24]. Individuals with prior cardiovascular disease had a higher homocysteine concentration than individuals without prior

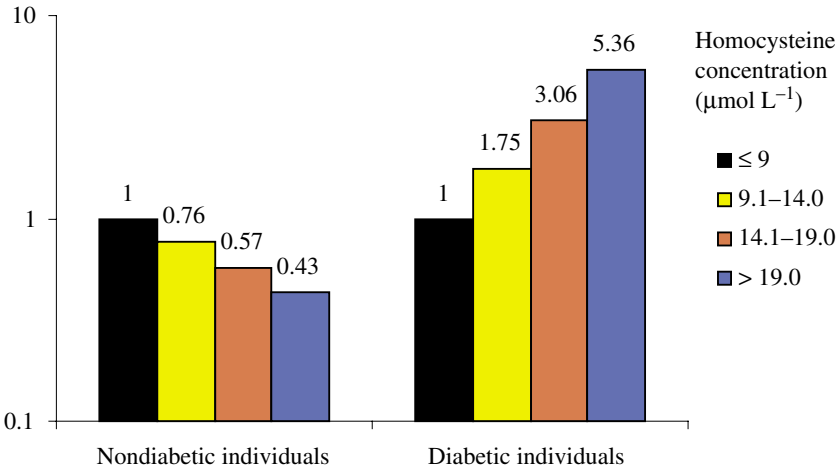

Fig. 1 Adjusted hazard ratios for coronary events amongst individuals with type 2 diabetes per category increment of homocysteine (≤9, 9.1–14.0, 14.1–19.0 and >19.0 μmol L^{-1}, respectively).

cardiovascular disease: 12.2 (9.7–15.6) μmol L^{-1} vs. 11.1 (9.2–13.8) μmol L^{-1}; *P*-value, 0.03.

Secondly, additional adjustment for diabetes duration, glomerular filtration rate or waist-to-hip ratio did not materially influence any of the associations (data not shown). Finally, we investigated whether individuals with a normal and an impaired glucose tolerance could reasonably be considered as one group. In stratified analyses, we found that hazard rates were similar for individuals with a normal and an impaired glucose tolerance (data not shown).

Discussion

The novel finding in this study is that homocysteine is a risk factor for coronary events in type 2 diabetes. We observed, in a population-based study with a 10-year follow-up period, a significant dose–response relation between homocysteine concentration and risk of coronary events. For each 5 μmol L^{-1} increment of serum homocysteine, the risk of coronary events rose by 28% (95% CI, 2–58).

Total homocysteine was a significant risk factor for coronary events in individuals with, but not without, type 2 diabetes. This is compatible with a recent meta-analysis, which has suggested that homocysteine is a relatively weak risk factor for coronary events [3]. In addition, studies suggest that homocysteine is most clearly associated with cardiovascular disease in high-risk populations [25–27]. Therefore, an association between homocysteine and coronary events may be clearest amongst individuals with type 2 diabetes, because they are at high risk of coronary heart disease. The biological mechanism for the interaction, if any, between homocysteine and type 2 diabetes is not clear. Hyperhomocysteinaemia may enhance atherothrombotic pathways through accelerating direct cytotoxic effects of glucose and through increasing glucose-induced oxidative stress on endothelial cells [5, 28]. Support for the latter hypothesis was recently provided by Shukla *et al.* who demonstrated that concentrations of homocysteine which had no effect on aortae from normal rabbits markedly reduced bioavailability of nitric oxide in aortae from diabetic rabbits, an effect that was fully reversed with superoxide dismutase [29].

In type 2 diabetes, homocysteine may be a risk factor not only for coronary events, but also for cardiovascular mortality [5], retinopathy [30] and microalbuminuria [31, 32]. In contrast, the association of homocysteine with micro- and macro-angiopathy in type 1 diabetes has not been extensively investigated, and this issue requires further study [33].

Long-term within-person fluctuations of homocysteine may lead to a systematic underestimation of the magnitude of the association between homocysteine and coronary events, especially after long follow-up periods, which is referred to as 'regression dilution bias' [19, 20]. The same phenomenon has been demonstrated for blood pressure and serum cholesterol [19, 34]. As suggested by Clarke *et al.* we adjusted hazard ratios for regression dilution to obtain a better estimate of the true strength of the

associations between average long-term homocysteine concentrations and disease risk [20]. The RDR calculated with data from a small sample of the Hoorn Study was comparable with the RDR of Clarke *et al.* [20] We decided to use the latter RDR, because this was calculated on the basis of four large studies.

Primary prevention is important in individuals with type 2 diabetes, especially because 50% of patients with type 2 diabetes may die within the first year after a first cardiac event [35]. As we observed that homocysteine was associated-coronary events in individuals with diabetes, lowering concentrations of homocysteine may be an attractive additional target for primary and secondary prevention in type 2 diabetes. Homocysteine concentrations can easily be lowered by daily supplementation with folic acid and possibly vitamin B_{12} [36]. However, results of large trials have to be awaited to know whether lowering homocysteine concentrations with vitamin B does indeed decrease risk of coronary events [37]. This study had several limitations. The population consisted of individuals aged 50–75 years, all belonging to caucasian population. Therefore, we do not know whether our findings can be generalized to younger or older individuals, or to other ethnic groups. Secondly, the associations we found between homocysteine and coronary events might have been affected by nondifferential misclassification, because events had to be classified according to specific causes. This generally results in an underestimation of the true effect [24]. Thirdly, results may be affected by information bias, because doctors may be more alert in detecting coronary events in diabetic patients, as they are already at a higher risk for coronary events than people without diabetes. However, this is the case in most follow-up studies. Our study lacked power to address associations between homocysteine and risk of other or more specific nonfatal cardiovascular endpoints, such as stroke and myocardial infarction. Finally, our data had insufficient power to firmly establish (or exclude) a threshold effect, and to assess whether the strength of the association of homocysteine concentrations with fatal coronary events differed significantly from that with nonfatal coronary events. These issues need to be investigated in a larger study.

In conclusion, we observed that total homocysteine concentration was associated with risk of coronary events amongst individuals with type 2 diabetes. In addition, other studies found that, amongst individuals with type 2 diabetes, homocysteine was associated with retinopathy [30] and microalbuminuria [31, 38]. Therefore, investigation of the effect of treatment with vitamin B on prognosis of individuals with type 2 diabetes is warranted.

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